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Application of Capillary Electrophoresis Laser Induced Fluorescence to the Detection of Nucleic Acid Probe Fragments

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APPLICATION OF CAPILLARY ELECTROPHORESIS- LASER INDUCED FLUORESCENCE TO THE DETECTION OF NUCLEIC ACID PROBE FRAGMENTS

by

C. A. BOULET, G. HUNG, and D. E. BADER

ABSTRACT

The Canadian Forces require rapid, sensitive systems for identification of bacterial and viral biological warfare (BW) agents in environmental matrices such as air and water. The system must detect BW agents at extremely low concentrations with no false alarms and operate under battlefield conditions. Gene probes can be used to target signature sequences of BW agents for identification; Cycling Probe Technology (CPT ™) is a gene probe identification technique based on the use of target nucleic acid as a catalyst for the conversion of chimeric probe molecules to detectable products. Capillary zone electrophoresis with laser induced fluorescent detection (CE-LIF) is an attractive technology for the detection of nucleic acid fragments because of its ultra-low detection limits and relatively simple instrument design. Together, CE-LIF detection of gene probe fragments offers the potential for developing a sensitive BW agent identification capability. In this work, a chimeric 5' $(DNA)_8(RNA)_4(DNA)_{16}$ 3' probe for *Bacillus globigii,* an anthrax simulant, designated as DRES2A, was used. In a typical experiment , the 5' fluoresceinated DRES2A probe (10 fmoles/ μL) and synthetic target DNA (10^{-4}-10^{-7} pmoles/μL) were incubated at 65°C for 30 min in the presence of RNase H. A 1:10 dilution of CPT reaction mixture was then analyzed by CE-LIF. The intact probe and cleaved 5' fluoresceinated-DNA product(s) were separated and detected using a Beckman P/ACE 5010 CE-LIF (ssDNA gel column, 7 cm L_d) in under 5 minutes. As low as 1attomole of target DNA in the CPT probe assay could be detected. This work demonstrates that CE-LIF can be used to separate and detect the products of the CPT assay and that CE-LIF can be a sensitive method for the detection of nucleic acid fragments.

EXECUTIVE SUMMARY

Title: Application of Capillary Electrophoresis-Laser Induced Fluorescence to the Detection of Nucleic Acid Probe Fragments

Authors: C. A. Boulet, G. Hung, and D.E. Bader

Introduction: The Canadian Forces require rapid, sensitive systems for identification of bacterial and viral biological warfare (BW) agents in environmental matrices such as air and water. The system must detect BW agents at extremely low concentrations with no false alarms and operate under battlefield conditions. Gene probes can be used to target signature sequences of BW agents for identification. Cycling Probe Technology (CPT ™) is a gene probe identification technique based on the use of target nucleic acid as a catalyst for the conversion of chimeric probe molecules to detectable products. Capillary zone electrophoresis with laser induced fluorescent detection (CE-LIF) is an attractive technology for the detection of nucleic acid fragments because of its ultra-low detection limits and relatively simple instrument design. Together, CE-LIF detection of gene probe fragments offers the potential for developing a sensitive BW agent identification capability.

Results: In this work, a chimeric 5' $(DNA)_8(RNA)_4(DNA)_{16}$ 3' probe for *Bacillus globigii*, an anthrax simulant, designated as DRES2A, was used. In a typical experiment, the 5' fluoresceinated DRES2A probe (10 fmoles/ μL) and synthetic target DNA (10^{-4}-10^{-7} pmoles/μL) were incubated at 65°C for 30 min in the presence of RNase H. A 1:10 dilution of CPT reaction mixture was then analyzed by CE-LIF. The intact probe and cleaved 5' fluoresceinated-DNA product(s) were separated and detected using a Beckman P/ACE 5010 CE-LIF (ssDNA gel column, 7 cm L_d) in under 5 minutes. As low as 1attomole of target DNA in the CPT probe assay could be detected.

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Significance of Results: CGE-LIF provides a sensitive and effective method for the detection of CPT reaction products to detect BW agents. CGE can detect the CPT reaction products significantly faster than previously implemented methods of CPT product detection, such as light addressable potentiometric sensors (LAPS) or electrophoretic separation of radiolabelled oligonucleotides. CGE requires less sample preparation, avoids the use of radiolabelled oligonucleotides, and yields reproducible migration times that can be used for identification. This work demonstrates the detection of attomolar quantities of target DNA in under 7 min on a gel-filled 7cm capillary. The low detection limit capability of LIF allows for much earlier detection of CPT reaction products, more rapid field identification, and ultimately earlier warning of BW agent attacks.

Future Work: The capillary electrophoresis-laser induced fluorescence method developed is suitable for the laboratory-based analysis of CPT reaction products. Further research will be required to develop a field portable or micronanalytical device based on capillary electrophoresis principles. CPT is one example of a gene probe method for the identification of biological agents. It remains to be seen whether this gene probe method will provide the speed, sensitivity or specificity required for biological warfare agent identification.

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1. INTRODUCTION

The goal of this research is to develop rapid, robust assays and equipment that are amenable to field detection and identification of BW agent attacks. The system must detect BW agents at extremely low concentrations with no false alarms and operate under battlefield conditions.[1] Typically high volume air samplers are required to concentrate particulate matter into liquid samples for analysis, often by a factor of 10^6.[2] To provide the earliest possible warning and maximum protection to personnel, the field detection and identification equipment (and associated assay chemistry) must be highly specific, ultra-sensitive, and very reliable.[3]

Capillary electrophoresis provides several advantages which are attractive for field-portable instrumentation. It has a simple, easily automated design, has very low volume requirements, and high separation efficiencies. Capillary gel electrophoresis (CGE) was selected for initial method development because it has been used for determination of oligonucleotide purity, PCR product and forensic DNA analysis.[4]

Cycling Probe Technology (CPT™) is a nucleic acid identification technology based on target nucleic acid acting as a catalyst for the conversion of chimeric probe molecules to detectable products.[5] The single-stranded (ss) DNA target serves as the catalyst for the CPT reaction. In the presence of excess chimeric probe and RNase H, the RNA portion of the resulting probe-target complex is cleaved by RNase H at 65°C. The shorter cleaved fragments dissociate from the target thereby regenerating the target DNA for further cycling. With each cycle, the released fragments accumulate and can be detected. CPT has certain advantages over other amplification methods such as polymerase chain reaction (PCR) that make it worthwhile investigating for field identification of BW agents. It is fast (≤30 min), simple (one probe, one enzyme, one temperature), and is not prone to false positives due to sample carry-over since the target DNA is not amplified.

CGE with laser-induced fluorescent detection (CGE-LIF) can provide the necessary specificity for the analysis of CPT products and identification of BW agents because of its ultra-low detection limits.[6][7] CE-based instruments are being developed to operate under field conditions.[8] This paper describes the fluorescent detection of the CPT reaction products by CGE-LIF as a possible method for BW agent identification.

2. MATERIALS AND METHODS

2.1. DESIGN AND CONSTRUCTION OF DRES2A CYCLING PROBE

The DRES2A cycling probe was developed for the detection of *Bacillus globigii* in a CPT assay.[9] The probe was designed to detect the division initiation gene of *Bacillus subtilis*, *div*IB, accession number M31800.[10] The DRES2A probe has the following construction, 5' Fluorescein- cgc ctg ta (aaaa) tgg atg aaa acc gta t-biotin 3'. The bracketed region denotes the ribonucleotide region of the probe. The DRES2A probe was purified by HPLC and polyacrylamide gel electrophoresis. The 5'-fluorescein label was employed for the LIF detection in this work while the 3'-biotin label was required for other studies.

2.2. CYCLING PROBE REACTIONS

CPT assay reactions were performed by ID Biomedical Corporation (Burnaby, B.C.) using procedures described elsewhere.[9] Reactions were promptly frozen following the CPT incubation step to stop the reaction and then thawed prior to analysis. CPT probe reactions were performed with either 10 fmol or 100 fmol of DRES2A probe. The assay concentrations for the synthetic target DNA (the complement of the DRES2A probe sequence) are given in the figures.

2.3. INSTRUMENTATION

CGE analyses were performed on a Beckman P/ACE System 5010, equipped with a P/ACE System Laser Module 488 (488 nm argon ion laser), and a P/ACE LIF Detector. Data was collected, stored, and analyzed using Beckman System Gold 7.0 software, Microsoft Excel 5.0a, and Grapher 1.28 on a Dell Optiplex 466/L.

2.4. ELECTROPHORETIC ANALYSIS

Electrophoresis of the single stranded chimeric CPT probes was performed using 37 cm Beckman eCAP ssDNA 100 Gel capillaries containing a fixed polyacrylamide gel. Beckman Gel Buffer solution, consisting of 44% Tris, 56% borate and 7 M urea, at pH 8.4, was filtered through a 0.22 μm filter and sonicated before use. A 37 cm length of eCAP ssDNA 100 capillary was installed into a Beckman P/ACE System LIF Detection Capillary Cartridge. Analyses were performed in either constant voltage or constant current modes, at 7.5 kV and 10.0 μA, respectively (30 °C).

2.5. SAMPLE PREPARATION

The CPT reaction solutions were thawed and an aliquot of each was diluted to provide a working volume (10 μL) for effective pressure or electrokinetic injection. The 100 fmol probe reaction solutions were diluted in a 1:10 ratio with 1 μL of original reaction solution being mixed with 9 μL of deionized distilled water. The 10 fmol probe reaction solutions were diluted in a 1:5 ratio, with 2 μL of original reaction solution being mixed with 8 μL of deionized distilled water.

2.6. CAPILLARY ZONE ELECTROPHORESIS OF OLIGONUCLEOTIDES

Two methods, both using simple fused silica capillaries and employing capillary zone electrophoresis (CZE) to separate oligonucleotide mixtures, have been recently published: one used a 300 mM borate and either a 25 mM or 50 mM SDS buffer (B-SDS), pH 9.0 [11]; the second used a 240 mM boric acid and 40 mM phytic acid, pH 9.1 buffer (BA-PA) [12].

Two oligonucleotides (pdA_8 and pdA_{28}), whose base lengths of 8 bases and 28 bases, represented the cleaved CPT probe product and intact probe lengths respectively, were synthesized on a Beckman Oligo 1000 oligonucleotide synthesizer (Mississauga, ON) in 30 nmole quantities according to the manufacturer's recommended procedures. Following cleavage and deprotection of the oligonucleotides from the solid column support using the Beckman Ultrafast (AMA) Cleavage and Deprotection Reagent™, the reagent was removed by evaporation on a Savant AES1000 SpeedVac (Savant Instruments, Farmingdale, NY) until dry (2 hrs) and then resuspended in 50 µL of sterile nuclease free water (Promega Corporation, Madison, WI). Concentrations were determined from spectrophotometric measurements at wavelengths of 260 nm using a Unicam 8700 UV/VIS Spectrometer (Unicam Ltd, Cambridge, UK) and a conversion factor of 1 OD unit corresponding to 40 µg/mL.[13]

3. RESULTS AND DISCUSSION

As the goal of this work is rapid identification, a modified CGE system was used to substantially reduce the analysis time. Initial experiments with a 30 cm L_d capillary column gave a migration time for the intact DRES2A probe of 18.5 min which was considered much too long for a practical field-identification method where near real-time identification is ideal. Since the L_d with cartridge-mounted capillary columns in the usual CE operating mode is 20 cm, much shorter capillaries were required to reduce the analysis time and provide faster detection. By changing the polarity and injecting the sample electrokinetically at the cathode (the outlet tray of the Beckman autosampler), a 7 cm L_d was possible and overall analysis times were substantially reduced.

A DRES2A CPT probe standard (1 fmol/μL) was used to establish reproducible CGE conditions. Initial experiments using constant voltage showed poor reproducibility of the 28 base DNA-RNA-DNA chimeric oligonucleotide's migration times. However the migration times were found to be more reproducible using a constant current of 10.0 μA rather than a constant voltage of 7.5 kV. With a 7 cm eCAP 100 ssDNA capillary column, the migration time for the intact DRES2A probe was 6.1 min (Fig. 1).

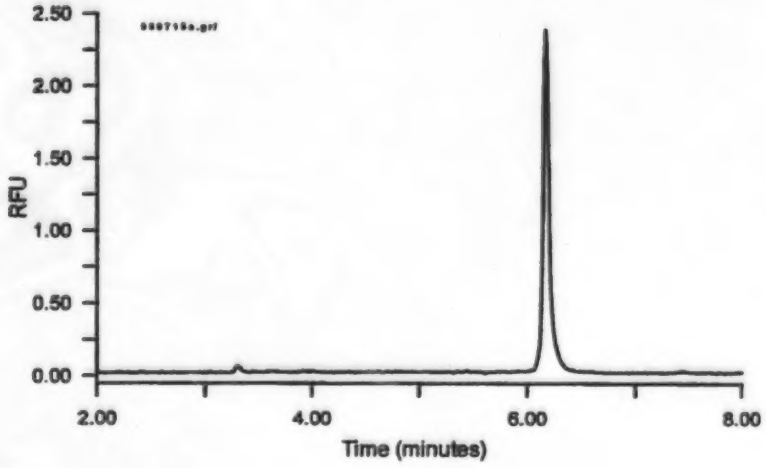

Figure 1. Electropherogram of DRES2A CPT probe standard (1 fmol/μL).

The analysis of the 100 fmol probe reaction solution with synthetic target clearly demonstrated the appearance of a shorter, faster-migrating fluoresceinated product at 4.3 min (Fig. 2) which was considered to be the 5' fluorescein-labelled fragment of the intact probe. The relative amounts of product to probe, corresponded to the expected trend that increased amounts of synthetic target in the reaction solution increased the proportion of the intact probe that was detected as the product fragment. The electropherogram of the 100 fmol probe CPT reaction solution with 10^{-6} pmol of target displayed the migration time of the reaction product at 4.3 min, with a small amount of the unreacted intact probe evident at 6.1 min (Fig.2A).

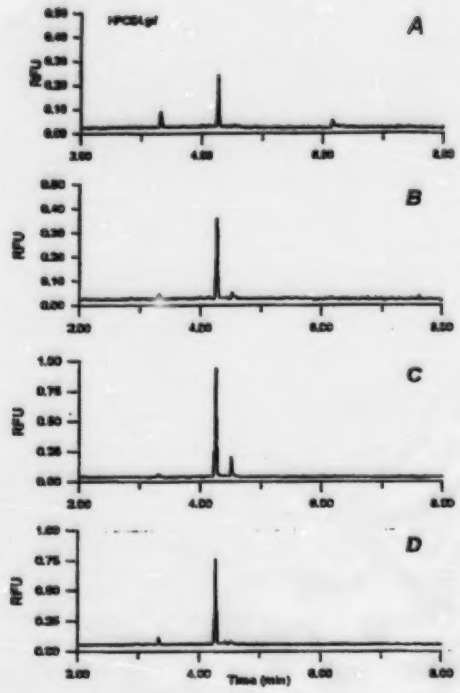

Figure 2. Electropherogram of 100 fmol DRES2A probe, 0.2 µg RNase H CPT reaction solution with 10^{-6} (A), 10^{-5} (B), 10^{-4} (C) and 10^{-3} (D) pmol of synthetic DNA target.

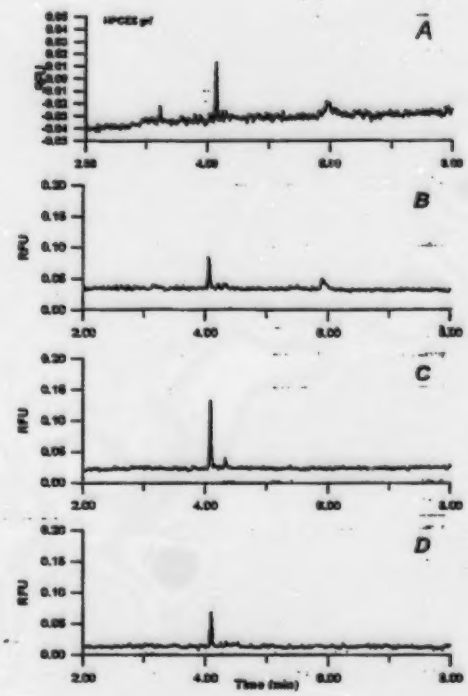

Figure 3. Electropherogram of 10 fmol DRES2A probe, 0.2 µg RNase H CPT reaction solution with 10^{-6} (A), 10^{-5} (B), 10^{-4} (C) and 10^{-3} (D) pmol of synthetic DNA target.

Decreasing the amount of probe from 100 fmol to 10 fmol in the reaction solution still produced a detectable amount of product for all target DNA concentrations tested (Fig. 3). As observed in the 100 fmol probe reactions, increasing the amount of synthetic target in the reaction solution increased the proportion of intact probe that was converted into cleaved product. All of the reactions essentially proceeded to completion with only trace amounts of intact probe evident.

CE analysis of the C2 control solution (probe + RNase H) indicated that the intact probe was cleaved in the absence of target DNA (Fig. 4). C2 activity is a phenomenon of the CPT reaction and occurs likely as a consequence of the probe acting as a substrate for the enzyme through the formation of hairpin structures (intramolecular interactions) or dimers (intermolecular interactions).[14] Interestingly, hairpin and dimer formation analysis of the DRES2A probe using Oligo v4.1 (National Biosciences, PLymouth, MN, USA) and Primer Premier v4.1 (Premier Biosoft International, Palo Alto, CA, USA) predicted no dimer formation and negligible hairpin formation.

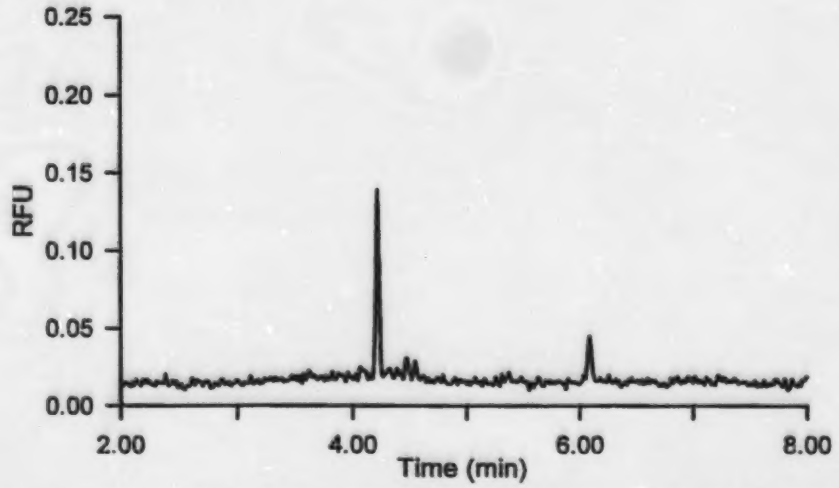

Figure 4. Electropherogram of the CPT C2 control solution (100 fmol probe plus 0.2 μg RNase H in CPT buffer).

Since proportionally more product was formed in the presence of target DNA than in the C2 control solution, the expected CPT reaction proceeded above the background product formation. The addition of heterologous DNA (non-target DNA) can increase non-specific cleavage of the probe even further.[15] Non-specific cleavage of the probe, such as that seen in the C2 control, presents problems for the detection of low amounts of target DNA by CPT. Additives such as EGTA and spermine can reduce non-specific cleavage, thereby reducing background signal and improving sensitivity. [15] The probe appeared to be stable in the CPT buffer in the absence of RNase H enzyme as no degradation products were observed (Fig 5.).

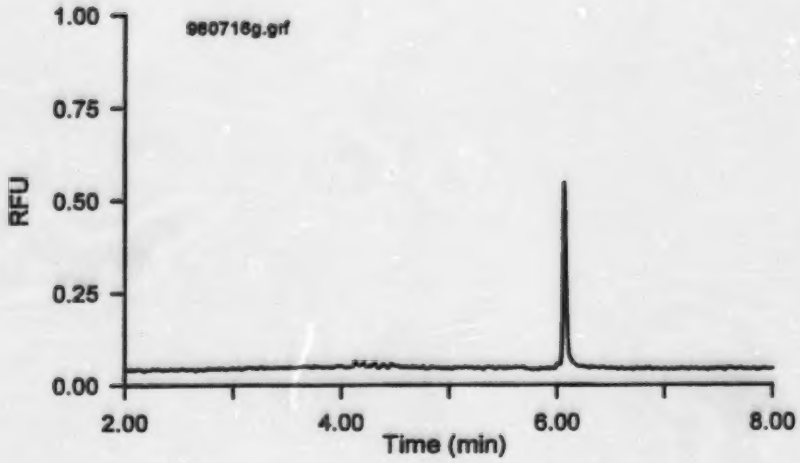

Figure 5. Electropherogram of the CPT C1 control solution (100 fmol probe in CPT buffer).

In an attempt to avoid the use of commercial, expensive gel-filled capillaries which are difficult to handle and easily dried out, alternative CE methods for oligonucleotide separation were examined. Two methods, both using simple fused silica capillaries and employing capillary zone electrophoresis (CZE) to separate oligonucleotide mixtures, have been recently published: one used a 300 mM borate and either a 25 mM or 50 mM SDS buffer (B-SDS), pH 9.0 [11]; the second used a 240 mM boric acid and 40 mM phytic acid, pH 9.1 buffer (BA-PA) [12].

Both methods rely upon the electroosmotic flow (EOF) to migrate the oligonucleotides along the capillary, according to each oligonucleotide's electrophoretic mobility. For the EOF to effectively migrate the species through the fused silica capillary, CE was performed as if the analyte was positively charged, and the Beckman P/ACE System was configured in the normal electrode mode. While the negatively charged ssDNA migrates towards the anode at the injection port, the EOF causes bulk flow of the buffer solution towards the cathode, and sweeps the ssDNA along with the EOF front towards the outlet port. Pressure injection was used exclusively, requiring that the longer 20 cm portion of a 27 cm fused silica capillary be used.

CZE was first attempted with the fluorescein-labelled oligonucleotide test mixes provided with the Beckman eCAP 100 ssDNA kits. CE analyses of the 1 fmol/μL DRES2A CPT probe standard using LIF detection and the B-SDS (25 mM SDS) buffer yielded questionable results, with a slight peak appearing after 4.5 min. These results are inconsistent with those observed in CGE, where the standard solution produced a large distinct peak. Injections of a 1:10 dilution of Beckman sodium fluorescein standard produced a sharp peak at 4.3 min, indicating that the fluorescent detection was operating. Separation of pdT 20- and 40-mers in the Beckman test kit was attempted in B-SDS (50 mM SDS), with a higher SDS concentration to increase the resolution capability . Two small peaks at 4.92 and 5.24 min roughly corresponded to the expected migration times for such oligonucleotides. A large peak is also observed at 1.65 min. A longer injection of this test mix did not increase the size of these two peaks. The same large peak was seen at 1.68 min in a B-SDS (50 mM SDS) buffer assay of the Beckman pdA 40-60-mer test mix. As with the previous test mix, no separation of the individual oligomers was observed.

An initial attempt of the CE analyses of oligonucleotide mixes using the BA-PA buffer was not successful. In the analysis of the Sigma 20-60mer Oligonucleotide Standard, a large peak was observed at 2.95 min, ahead of the EOF front at approximately 5.0 min. A much larger peak was observed later at 17.58 min, most likely due to the unseparated oligonucleotide mix. This was not unexpected, as it was reported that the BA-PA buffer system has poor resolving capabilities for oligonucleotides longer than 20 bases.

Two oligonucleotides (pdA_8 and pdA_{28}), whose base lengths represent the cleaved CPT probe product and intact probe lengths respectively, were synthesized. CE of the pdA_8 oligonucleotide using BA-PA buffer produced a broad peak at 2.93 min, as well as a sharp peak at 9.71 min (Fig. 6A); the electropherogram of the 28 base oligonucleotide (Fig. 6B) also produced the broad peak at 2.91 min, and a large peak at 19.21 min followed by a short broad peak at 22.00 min. The presence of several minor peaks for each synthetic oligonucleotide solutions is likely due to oligonucleotide fragments resulting from incomplete synthetic coupling.

The longer 28-mer, pdA_{28}, displayed more fragments owing to the greater number of synthesis cycles that is required. A separation of the two oligonucleotides (Fig. 6C) clearly shows both the 8-mer peak at 10.1 min and the 28-mer peak at 19.2 min, along with the oligonucleotide fragment peaks. These results demonstrate that it will be possible to separate the shorter 5' CPT probe fragment from the the longer intact probe using the simpler and less expensive CZE method.

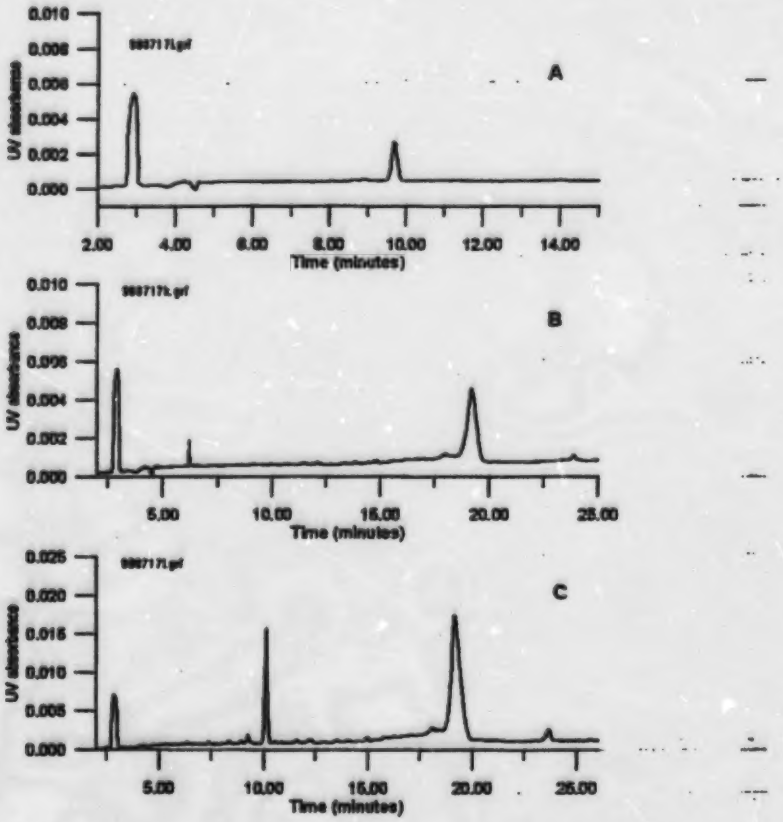

Figure 6: CZE analysis of two synthetic oligonucleotides, pdA_8 (A), pdA_{28} (B), and co-injection of both oligonucleotides using a 240 mM boric acid, 40 mM phytic acid separtion buffer at pH 9.1 (27 cm fused silica capillary, 50 μm id, L_d 20 cm, 10 sec pressure injection).

4. CONCLUSIONS

CGE-LIF provides a sensitive and effective method for the detection of CPT reaction products to detect BW agents. CGE can detect the CPT reaction products significantly faster than previously implemented methods of CPT product detection, such as light addressable potentiometric sensors (LAPS) or electrophoretic separation of radiolabelled oligonucleotides, which typically require much longer analysis times. CGE requires less sample preparation, avoids the use of radiolabelled oligonucleotides, and yields reproducible migration times that can be used for identification. This work demonstrates the detection of attomolar quantities of target DNA in under 7 min on a gel-filled 7cm capillary. The low detection limit capability of LIF allows for much earlier detection of CPT reaction products, more rapid field identification, and ultimately earlier warning of BW agent attacks.

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The Canadian Forces require rapid, sensitive systems for identification of bacterial and viral biological warfare (BW) agents in environmental matrices such as air and water. The system must detect BW agents at extremely low concentrations with no false alarms and operate under battlefield conditions. Gene probes can be used to target signature sequences of BW agents for identification; Cycling Probe Technology (CPT™) is a gene probe identification technique based on the use of target nucleic acid as a catalyst for the conversion of chimeric probe molecules to detectable products. Capillary zone electrophoresis with laser induced fluorescent detection (CE-LIF) is an attractive technology for the detection of nucleic acid fragments because of its ultra-low detection limits and relatively simple instrument design. Together, CE-LIF detection of gene probe fragments offers the potential for developing a sensitive BW agent identification capability. In this work, a chimeric 5' $(DNA)_8(RNA)_4(DNA)_{16}$ 3' probe for *Bacillus globigii*, an anthrax simulant, designated as DRES2A, was used. In a typical experiment , the 5' fluoresceinated DRES2A probe (10 fmoles/µL) and synthetic target DNA (10^{-4}-10^{-7} pmoles/µL) were incubated at 65°C for 30 min in the presence of RNase H. A 1:10 dilution of CPT reaction mixture was then analyzed by CE-LIF. The intact probe and cleaved 5' fluoresceinated-DNA product(s) were separated and detected using a Beckman P/ACE 5010 CE-LIF (ssDNA gel column, 7 cm L_d) in under 5 minutes. As low as lattomole of target DNA in the CPT probe assay could be detected. This work demonstrates that CE-LIF can be used to separate and detect the products of the CPT assay and that CE-LIF can be a sensitive method for the detection of nucleic acid fragments.

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Cycling Probe Technology

CE-LIF

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oligonucleotide analysis

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